SOME CONSIDERATIONS OF THE BIOLOGICAL IMPORTANCE OF INTESTINAL MICROÖRGANISMS[1]

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The relationship of intestinal bacteria to the well-being of the host has been of general interest for over sixty years. Despite numerous studies conducted to determine the rôles of these organisms in health and disease, their functions are not completely understood. The kinds of bacteria found in the digestive tract and their distribution have been extensively studied in many animal species including man. It is known that the ecology of the intestines is very complex; changes which have been found to occur in the numbers and kinds of microörganisms in the intestines are difficult to explain.

Nutritionists, bacteriologists and physicians now tend to regard intestinal microörganisms as sources of nutrilites indispensable to the animal host, particularly if the diet is poorly balanced; previously these organisms had been considered only as potential causative agents of gastro-intestinal disturbances. Many of the early investigators considered intestinal bacteria as saprophytes whose putrefactive metabolic products were harmful. Later, it was realized that there are two main types of intestinal bacteria: acid-producing or saccharolytic species, and proteolytic species. The latter group always has been considered harmful or undesirable, but their true significance is not settled. Acid-producing species, on the contrary, always have been deemed desirable for proper conditions in the intestinal tract. Recently, with the help of the rapid advances made in the field of nutrition, considerable evidence has been accumulated which indicates that intestinal bacteria are important in supplying essential growth substances to the host, although it is not certain that they are indispensable. Earlier work now can be re-evaluated with more assurance of drawing valid conclusions. However, there still exists a variety of viewpoints which may eventually be consolidated when more basic work is accomplished.

Probably the first observation of intestinal microörganisms was made by Leeuwenhoek (17) in about the year 1674. Many of the organisms he saw with his simple microscope no doubt were bacteria. Two centuries later, Escherich initiated what is considered to be the first comprehensive study of intestinal bacteria in human infants, and laid the foundation for future work in this field.

THE RÔLES OF INTESTINAL BACTERIA

The present tendency of modern nutritionists to think of intestinal microorganisms in terms of their nutritional importance to their host is based on a

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series of investigations which began with pure speculation by Pasteur. Today it is postulated that intestinal bacteria actually synthesize essential growth factors within the lumen of the gut. In fact, it has been stated by Teply *et al.* (97) "That vitamins are synthesized by intestinal microörganisms has been proved beyond any reasonable doubt."

Following Pasteur's (75) meditations concerning the necessity of microorganisms for life, numerous studies were instituted to shed some light on this highly controversial problem. Metchnikoff (60) was the proponent of the idea that the activities of intestinal bacteria are directly correlated with longevity of the host; a flora consisting mainly of lactic acid bacilli is supposed to subdue proteolytic activities of other microbes, resulting in a more vigorous, longer-lived individual. He had many adherents and some very good evidence to support his beliefs (see Rettger and Cheplin, 81, for a detailed review). Another line of attack, one that is now being pursued more actively than ever, involved attempts to raise various animals with a bacteria-free intestinal tract. It would seem that this type of study should result in a positive conclusion concerning the necessity of intestinal microörganisms for life, or at least, for the general well-being of the host. An exhaustive review of this subject has been presented by Glimstedt (33), hence an extensive discussion is not believed necessary. Nuttall and Thierfelder (71, 72) were the first to raise animals in a sterile environment. They observed impaired growth in some of the guinea pigs raised under aseptic conditions for 13 days. Later, Schottelius (87) and Cohendy (12) succeeded in keeping chicks free from microörganisms for varying lengths of time. Schottelius noted that his bacteria-free chicks grew more slowly and did not survive as long as normal chicks consuming the same sterile ration. Cohendy could find no significant difference between bacteria-free and normal chicks. Thus, the two workers obviously drew different conclusions from their observations. That many of the early failures encountered in raising healthy animals under conditions which would exclude intestinal microörganisms were a result of meager knowledge of nutritional requirements cannot be doubted in view of present-day advances in the nutrition field. Many of the early failures provided excellent evidence for the intestinal synthesis of growth factors. Modern attempts to raise bacteria-free higher animals apparently have been more successful (82, 83), though no conclusive proof of the adequacy of the nutrition of these animals has been established.

The "modern" viewpoint of the rôle of intestinal bacteria is that they provide the host with essential nutrilites. This so-called "modern" viewpoint probably originated with the studies of Osborne and Mendel (73) who attempted to raise rats on highly purified rations containing a single protein, carbohydrate, and fat, plus a salt mixture and agar-agar; these rations now are considered to be highly deficient in essential fractions of the vitamin B complex. They noted the tendency of these rats to become coprophagous, and that this practice alleviated the growth-depressing effects of their various vitamin-deficient diets. When these rats were allowed to choose between their own feces and those from rats on an adequate ration, they invariably chose the latter. Osborne and Mendel believed that the superiority of feces from normal rats could be attributed to the bacterial flora which, as had been pointed out by Herter and Kendall (41), was markedly influenced by the composition of the diet.

Rettger and Horton (80) and Hull and Rettger (42) coöperated with Osborne and Mendel by performing bacteriological studies on feces from rats fed complete and incomplete rations. A considerable difference was noted between the characteristic flora of rats maintained on the simple "vitamin B" deficient diet and the flora of those on a stock laboratory ration; a simple flora composed of predominately gram-positive bacilli (in some cases nothing but gram-positive bacilli were evident) typified rats ingesting the simple diets of Osborne and Mendel, whereas rats consuming a more adequate ration maintained a more complex fecal flora. They found coliforms to be low in numbers in droppings from rats on either of the two types of diets, but these organisms were particularly scarce in rats on deficient diets. These investigators noted that feeding rats a high milk diet, or a ration high in lactose, had the same effect in simplifying the fecal flora as did feeding the purified, deficient diets of Osborne and Mendel. The gram-positive bacilli found in rat feces were identified as *Lactobacillus acidophilus* and *L. bifidus*.

The earliest suspicions that intestinal synthesis was related to "vitamin B" were stated before this vitamin was shown to be made up of many fractions. Evidence for synthesis was determined by feeding feces of one animal to another animal deficient in "vitamin B." The availability of the synthesized vitamin in the feces to the animal was not considered to any great extent until a number of years later and still remains a controversial issue. Cooper (13) appears to have been the first to suggest actual synthesis of a vitamin by intestinal bacteria. He was able to alleviate polyneuritis in pigeons fed polished rice by feeding them alcoholic extracts of feces of hens and rabbits kept on normal diets. He believed that the anti-neuritic substance was synthesized by intestinal bacteria in the hens and rabbits. This work was repeated subsequently by Portier and Randoin (77) and similar results were obtained. A similar study was made by Theiler and his associates (99) which implicated bacterial synthesis in the rumen of cattle. They believed that a troublesome South African paralytic disease, "lamzietke," might be the result of a nutritional deficiency. In an attempt to reproduce the disease by controlled feeding experiments, they observed that a ration deficient enough in "vitamin B" to produce polyneuritis in pigeons was in every way adequate for cattle. Their assumption was that either cattle required very little "vitamin B," or that intestinal microörganisms synthesized the deficient vitamin, which in turn was utilized by the animal; the latter seemed the more reasonable view. This work opened up the whole new field of rumen synthesis, consideration of which is beyond the scope of this review, although a large share of our knowledge of biosynthesis has been derived from such studies. It is suggested that the reader consult the excellent reviews by Hastings (39), Thaysen (98), Smith (91), and Kon and Porter (47) for further information on rumen synthesis.

Steenbock and co-workers (93) demonstrated more completely the importance of coprophagy to the rat. They noted that when rats on an apparently normal grain ration were deprived of the opportunity to consume their own feces by the use of raised, wide-meshed screen-bottomed cages, they developed a "vitamin B" deficiency. On this particular ration they calculated that coprophagy supplied over 50 per cent of the "vitamin B" requirement of the rats. Dutcher and Francis (19) confirmed and re-emphasized the findings of Steenbock, *et al.* Heller, *et al.* (40) more directly implicated bacteria as the source of B vitamins furnished in the feces. They observed that rats on a vitamin-deficient diet grew normally if allowed to consume their own feces, and found such feces to contain considerably larger numbers of spore-forming bacteria than feces from rats unable to engage in coprophagy.

In 1927 Fridericia, *et al.* (28) and Roscoe (85) independently published papers which undoubtedly influenced future approaches to nutritional studies. They described spontaneous recoveries of rats which had been depleted on a vitamin B-complex deficient ration. Such recoveries occurred when the rats passed bulky white feces with a high starch content. Fridericia, *et al.* proposed a new term, "refection," to denote this unusual process. It was concluded that the spontaneous recoveries observed in their work were due to consumption of refected feces. The protective phenomenon could be transmitted from animal to animal by feeding feces from the refected animals. Fridericia showed that the agent in the feces was thermolabile and did not pass through a Berkefeld filter. Raw starch was found essential for refection (cooked starch was inactive) although dextrinized corn starch, in certain cases, was found to produce the same effect; sucrose was not beneficial. Fecal contents of refected rats were more acid than those of non-refected rats and showed a different bacterial flora. The hypothesis, therefore, was advanced that "vitamin B"-producing microörganisms had become established in certain animals on a vitamin-deficient diet which had brought about refection. Roscoe emphasized the necessity for control of coprophagy in such nutritional studies in stating: "Whatever construction of cage is used, there are some rats that cannot be prevented from eating their own feces, for they secure them and consume them as they leave the body."

The interesting point concerning these studies on refection was the finding that starch was necessary in the diet for the manifestation of refection. Bliss (8) corroborated these earlier discoveries but was much more successful in producing refection in rats by incorporating raw potato starch in the ration as a substitute for the rice starch used by Fridericia, *et al.* and by Roscoe. Bliss was not too successful in producing refection with either raw rice or corn starches. He demonstrated that the starch was not the source of the B vitamins. Bliss's results showing a differential action of the three different starches may have some relation to the work of Langworthy and Deuel (51), who studied the degree of assimilation of raw corn, wheat and potato starches in three young, healthy men by determining the amount of starch remaining in their stools. Corn and wheat starches were entirely digested, whereas potato starch was found to be from 62.3 to 95.2 per cent digested (average of 78.2). Corn and wheat starches caused no complaints from the subjects, but potato starch caused excessive gas formation with abdominal cramps. Apparently, the differential effect of various dietary starches has been ignored by nutritionists in attempting to explain the phenomenon of refection. According to Coates *et al.* (11), evidence which appears to explain the cause of refection is accumulating, and seems to indicate that the amount and kind of carbohydrate available to intestinal bacteria influences the degree of vitamin synthesis as well as the qualitative nature of the flora. Fridericia (46) had mentioned this as a problem worthy of more consideration. Investigations elucidating the rôle of carbohydrates in nutrition and their effects upon the intestinal flora will be reviewed in the next section of this report.

Although, as previously pointed out, several of the early nutritionists obtained evidence which was indicative of an elaboration of growth factors by intestinal microörganisms, it remained for Bottomley (9) to observe actual production of a nutritive factor by bacteria. He noted that incubation of peat with a mixed culture of aerobic soil bacteria ("bacterized peat") resulted in stimulation of wheat seedling growth in this peat. This growth-promoting substance could be concentrated by chemical procedures similar to those used in purification of thiamine. Bottomley used wheat seedlings and *Azotobacter chroococcum* for testing his fractions; Najjar and Barrett (68) state that this is "probably the first instance of microbiological vitamin assay." Bottomley's work no doubt gave added encouragement to those who believed or suspected that intestinal microorganisms synthesized essential growth factors which were utilized by the host. However, it was not until the report of Pacini and Russell (74) that evidence was secured showing formation of a substance capable of stimulating animal growth. They showed that a water extract of the typhoid bacillus produced a marked growth response in rats ingesting a "vitamin B"-deficient ration. Similar results were reported subsequently by Bierry and Portier (7) who observed production of animal growth substances by unidentified bacteria which they called "symbiotes."

Many reports of synthesis by bacteria and the needs of bacteria for vitamins required by animals were made in the following years (see reviews 45 and 76) until it became generally accepted that these lower forms of life possessed certain metabolic systems similar to those of higher plants and animals (92). The work of Burkholder and McVeigh (10) and Thompson (100) conclusively pointed to the fact that "obligate" intestinal bacteria are able to synthesize many of the B vitamins required by animals. However, there were indications that certain of these vitamins might not be so readily available to the host because they were retained within the bacterial cells. This effect was also noted by others (1, 65). Also, when one considers the notably poor synthesizing capabilities of certain bacteria, e.g. lactobacilli, enterococci, and clostridia, all commonly encountered in the intestinal tract, it is apparent that a sort of competitive equilibrium must exist within the complex biological system of the digestive tract. An informative study by Benesch (4) resulted in the discovery that the mixed bacterial flora from a human appendix synthesized a considerable amount of nicotinic acid under aerobic conditions, whereas anaerobic incubation brought about destruction of this vitamin. He says, "It seems that normally there exists in the bowel an ecological system in which aerobic and anaerobic organisms live together, with the result that an equilibrium is constantly struck between the activities of the organisms producing and destroying nicotinic acid."

The prophetic work, which has been mentioned, of Fridericia, *et al.*, and of Roscoe, introduced a new concept in the field of nutrition. Their observations on refection intimated that synthetic factors produced by intestinal bacteria were released in the lumen of the intestine, and that from this source they could be absorbed and utilized by the host. Thus, refection predisposes to absorption of growth factors elaborated by intestinal microörganisms. The initial concept of refection was limited to the rat maintained under specific conditions. It was soon realized that refection was merely a special manifestation of the value of intestinal synthesis to the host. In other words, even without true refection, the host animals might benefit from synthesis of vitamins by intestinal bacteria; this is particularly true in ruminants.

With the discovery of refection, together with the earlier observation of the effects of coprophagy and its value to experimental animals consuming a vitamin-deficient ration, a wealth of indirect evidence rapidly began to accumulate pointing toward the value of intestinal synthesis to animals. The ruminant is unique in that the seat of vitamin synthesis is located in the anterior portion of the digestive tract thereby permitting more efficient absorption of the growth factors synthesized, and thus eliminating the necessity of coprophagy. With non-ruminant animals such as the rat (upon which more nutritional studies have been made than on any other animal), it is a question of the ability of the animal's gut to absorb such factors as may be synthesized before the feces are expelled from the body. Therefore, when an adequate ration is available, intestinal synthesis may act only in a supplementary capacity.

It has been shown by some workers (2, 50, 70) that there is considerable synthesis of vitamin K, riboflavin and biotin in the feces of rats and chickens after expulsion from the body. Recently, Kennard and Chamberlin (44) have reported upon the desirability of allowing floor-litter in chicken houses to accumulate so as to be an available source of some of the vitamins (including the poorly understood animal protein factor). These studies have a further bearing on the rôle of coprophagy in animal nutrition.

A series of studies on the rat by Guerrant and his co-workers (35, 36, 37, 38) has indicated that a large proportion of synthesized riboflavin, thiamine, and the undifferentiated B-complex in general is passed out of the body before these vitamins can be absorbed by the intestinal epithelium. Their results pointed to the cecum and colon as the likely areas for the greatest synthesis. They showed that a cecectomized rat was unable to synthesize sufficient vitamins to grow normally, and required supplements of baker's yeast or feces from rats on an adequate ration. An extremely high yeast population was noted in the cecum, and this led Guerrant, *et al.* to suggest that yeasts were responsible for biosynthesis. Their work also included a study on the differential effect of various carbohydrates upon growth. This subject will be reviewed further in the next section.

Various other studies have been conducted to determine the site of vitamin synthesis and the extent to which the vitamins are available to the host. A study by Mayhew (58) on the effect of removal of the ceca on the establishment and severity of cecal coccidiosis in chickens showed that uninfected, cecectomized birds on a normal grain ration grew as well as normal, uninfected chicks, except during the first post-operative week, and that removal of the cecum did not in any way impair egg production.

Griffith (34) studied the vitamin "B" and "G" requirements of cecectomized rats and reported only slight differences between his experimental and control animals. Taylor, *et al.* (96) believed that the basal diet Griffith used "may not have been favorable to the maintenance of bacterial growth in the cecum." They found that the cecum of the rat plays a significant rôle in vitamin synthesis only when the animal is maintained on an inadequate diet. They as well as Griffith noted a compensatory effect in their cecectomized rats, however, in that the diameter of the upper colon of operated rats had increased in size. Feces of normal rats fed a sub-optimum "diluted Purina ration" contained more folic acid, pyridoxine, and pantothenic acid but, for no apparent reason, less thiamine. Day, *et al.* (16) produced evidence which indicates that the cecum of the rat may be the main site of vitamin K synthesis as well as for absorption of this fat soluble vitamin. However, their results indicated that the cecum was not the only location for the synthesis of vitamin K since cecectomized rats on a ration deficient only in vitamin K grew well and evidenced no vitamin K deficiency unless one per cent sulfasuxidine was incorporated in the ration; rats with intact ceca on the same ration with sulfasuxidine evidenced no vitamin K deficiency. These results are indicative of the synthesis of vitamin K by bacteria other than those of the coliform group since numerous workers have shown that certain sulfonamides inhibit proliferation of this group of microörganisms (25, 32, 53, 56, 62, 66, 78). Schweigert, *et al.* (88) found that cecectomized rats on complete synthetic rations grew as well as the unoperated controls. Another report by McGregor *et al.* (59), indicates that the sites of biotin synthesis in the rat are in the cecum and large intestine.

That the cecum of the rat is different in certain respects from any other segment of the intestinal tract has been pointed out by Bergeim and colleagues (5, 6). They found the oxidation-reduction potentials to decrease markedly from the anterior to the posterior portions of the gastro-intestinal tract. They obtained E_h values as follows: stomach, $+150$; upper small intestine, -100; lower small intestine, -100; and cecum, -200 millivolts. Thus, the greatest reducing activity is located within the cecum. They observed an additional interesting effect: lactose, unlike any of the other carbohydrates used, resulted in such a decrease of reducing intensities that positive E_h readings were obtained throughout the intestinal tract for as long as 36 days in rats consuming a lactose ration. This revealing discovery of the anti-reducing effect of milk sugar should provide a stimulus for further research when one considers the effect of lactose on the intestinal flora (81) and the requirement of the young of all mammals for milk. Bergeim and his collaborators also noted that as E_h values became more negative from the stomach to the cecum, pH values of the intestinal contents increased.

No conclusive proof concerning the main locus of vitamin elaboration in all animals has thus far been advanced. However, several highly enlightening studies have been published which bear mention. Leong (52) reported destruction of vitamin B_1 in the rat, since tissue storage of this nutrilite was extremely low, even when B_1 was used in high concentrations in the ration. Abdel-Salaam and Leong (1) demonstrated synthesis of thiamine by a mixed culture of bacteria from the ceca of rats. They inoculated a thin suspension of cecal contents into broth and after varying lengths of time, the cells and the medium were analyzed for thiamine. They found the most thiamine present in the cells and medium after one day's incubation at 37 C; however, by far the most vitamin was found to remain within the cells, and beyond one day there appeared to be destruction of thiamine. Bacteria which were known to be involved in this study were enterococci, lactobacilli, clostridia and *Escherichia coli.* Likewise, Tange (95) reported the production of riboflavin by cecal bacteria of the rat. Shourie and Swaminathan (89) reported that rats on low and high nicotinic acid-containing diets excreted approximately the same amount of nicotinic acid in their feces. On low nicotinic acid intakes (5 to 22 μg per day), as much as 60 μg per day were excreted while on high nicotinic acid intakes (1,250 μg per day), about 85 μg per day were excreted. The nicotinic acid content of the liver, muscles and blood were strikingly similar in rats on the two diets with highly different amounts of this factor. Hence, there appears to be a certain amount of destruction or binding of nicotinic acid if present in large concentrations.

The destruction of various vitamins by bacteria *in vitro* is now a well established fact. The classical example is that of decomposition of vitamin C by common intestinal bacteria (106, 107). Koser and Baird (49) isolated 26 cultures from soil and water which had the ability to use nicotinic acid as the sole source of carbon. Microbiological destruction of riboflavin, pantothenic acid and thiamine has been reported (27, 61, 101). Koser's (48) discussion of "Growth Factor Destruction" elaborates further on this question. A further investigation by Reid (79) has shown indirectly that ascorbic acid when injected intraperitoneally in the guinea pig is found in considerable amounts in the contents of the stomach and small intestine, but appears in significantly smaller concentrations in the cecum and large intestine. This evidence appears to indicate that destruction of vitamin C occurs in the cecum and large intestine of the guinea pig, and that such destruction may probably be attributed to the microbial population of the intestines. Furthermore, it seems plausible that intestinal destruction of certain other of the known vitamins can and does occur. This, no doubt, will be revealed by experimental work in the near future. It is to be hoped that more direct means will be employed to prove the occurrence of microbial destruction of vitamins in the gastro-intestinal tract of mammals.

It has been demonstrated, both *in vivo* and *in vitro*, that bacterial destruction of a vitamin does occur and hence this activity may play an important rôle in the vitamin balance of the animal host. Furthermore, it has been indicated that vitamin synthesis in the intestinal tract is highly susceptible to the composition of the ration (this will be elaborated upon further in the next section) and may proceed to greater or lesser degrees depending on the particular location within the tract. Additional evidence of the delicate balance of the biological systems occurring in the intestines was compiled by Martin (57) and Woolley (102). The former noted that when the vitamin B-complex of a synthetic ration was supplied as riboflavin, thiamine, nicotinic acid, pyridoxine, choline and Ca pantothenate, mice grew well. If para-aminobenzoic acid (PABA) was included in the ration, the mice developed a deficiency syndrome not unlike that of a pantothenic acid deficiency; this deficiency could be corrected by the addition of inositol to the diet. Conversely, if inositol was incorporated in the ration, a PABA deficiency developed. Martin postulated that inositol may stimulate proliferation of pantothenate-destroying microörganisms and that PABA may inhibit them. Woolley, also working with mice, found that an inositol-free synthetic ration supported good growth of most of his animals. However, in the absence of pantothenic acid the mice developed alopecia (loss of hair), a manifestation of inositol deficiency. The inositol-synthesizing mice yielded mixed cultures from their droppings which were able to form considerable amounts of inositol—enough to bring about recovery of mice affected with alopecia. On the other hand, such mixed cultures as were isolated from the deficient mice synthesized very little inositol. An isolate of *Escherichia coli* was found to synthesize very little inositol and the presence of gramicidin (an inhibitor of gram-positive bacteria) had no effect on the degree of synthesis by mixed cultures from alopecia-free mice.

A further factor that may have to be considered in future nutritional studies is that of the rôle of vitamin analogues (84, 103, 104, 105). A recent report by Dreizen *et al.* (18) has shown that antimetabolites of nicotinic acid, pantothenic acid, and thiamine are able to depress growth and acid production of *Lactobacillus acidophilus* in a synthetic substrate. However, little is known of the natural occurrence of vitamin analogues and until this is determined, such studies may have to be deferred.

An excellent study by Mitchell and Isbell (65) has shed some light upon the degree of vitamin synthesis in the ceca of rats, the site of absorption, and the availability of certain B-complex vitamins. They studied synthesis of thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, biotin, inositol, and folic acid in rats eating lean beef with and without lactose. By removing the ceca of rats, centrifuging the contents and thus separating solids from the liquid, they were able to determine the amount of binding of the various vitamins within the bacterial cells. They found that inositol, thiamine, riboflavin, nicotinic acid and pantothenic acid were tied up in the cells to a considerable extent whereas the other members of the B-complex studied would readily diffuse from the cells into the medium. To determine the site of absorption, they took sections of the saline-washed walls of the stomach, upper small intestine, lower small intestine, cecum and colon, ground them immediately upon removal, and extracted them with boiling water. These water extracts were then analyzed for certain B vitamins (folic acid, biotin and pantothenic acid). They found that there was absorption of these B vitamins at every level of the tract but that it was considerably greater in the walls of the cecum. Since the amounts of B vitamins in the cecal wall extracts were similar in magnitude to those values obtained for the medium surrounding the bacteria, they believed that there exists a more or less free passage of vitamins from bacteria in the cecum into the animal tissues. The action of the two diets differed in numerous respects, the main one being that the diet with lactose resulted in a slightly greater synthesis of most of the vitamins in the cecum. From their extensive study, Mitchell and Isbell calculated the approximate percentages of the dietary intake of B vitamins supplied by intestinal synthesis: thiamine, 8 to 18; riboflavin, 5 to 19; nicotinic acid, 1.8 to 3.5; pantothenic acid, 11 to 58; pyridoxine, 130 to 230; biotin, 230 to 430; inositol, 0.08 to 0.1; and folic acid, 67 to 71 per cent. Figures over 100 per cent indicate that more of the vitamin was synthesized than was ingested. Apparently, certain synthesized vitamins are more available to the animal than others according to this study. Unfortunately, Mitchell and Isbell did not attempt a study of more than two rations, but one might anticipate that the availability of the various B vitamins, *i.e.* the degree of retention within the bacterial cells, might vary considerably according to the ration employed, and the presence or absence of carbohydrate.

Gall and her co-workers (29, 30, 31) have reported on a study involving two highly inbred strains of mice differing in their degree of requirement for riboflavin and pantothenic acid; strain C_{57} is black and possesses a high amount of resistance to mammary tumors whereas strain A is albino and is very susceptible to mammary tumors. Strain C_{57} was found to require less riboflavin and pantothenic acid in its diet than the albino strain. These workers felt that this nutritional difference in the two strains of mice might be reflected in unlike bacterial floras, and that members of the flora from the two strains of mice might show a great enough difference in their respective abilities to synthesize the vitamins in question that such a difference could explain the dissimilar nutritive needs of the mice. In report II, they studied the predominant bacteria from the lower small intestine and cecum of the two strains of mice (they believed that the lower bowel is the likely area for absorption of synthetic products liberated by bacteria) on three rations: a stock ration of Purina Laboratory Chow; ration 101, an incomplete synthetic ration with dextrose as the carbohydrate; and ration 133, which was more complete than 101, and incorporated dextrin as the sole carbohydrate. They found each ration to result in a characteristic bacterial flora in the lower intestines consisting of one or more specific morphological types. The stock ration resulted in the highest bacterial counts in the cecum; the ceca of animals on this diet were larger. In their third paper, they reported isolation of bacteria from the highest three dilutions of the intestinal contents in order to obtain representatives of the predominating types and to determine their ability to synthesize riboflavin, niacin, biotin, folic acid and pantothenic acid. No cultures were identified except coliforms and their methods of assaying for synthesis of the above-mentioned vitamins were semi-quantitative in nature. Hence, no revealing conclusions could be drawn. In their final paper, a basis for explaining the nutritional differences in the two strains of mice was reported. On a purified ration, it was found that the total weight of the cecal contents of the two strains was significantly different, although the bacterial count per gram of cecal material was essentially the same for both mouse strains. Strain C_{57} had the greatest total amount of cecal contents and strain A the least. On this basis they postulated that C_{57} actually possessed the largest total numbers of cecal bacteria and therefore greater amounts of vitamins were synthesized and absorbed in mice of this strain than in strain A. This explained their results admirably inasmuch as strain C_{57} possessed the lowest requirement for riboflavin and pantothenic acid. When the two strains of mice were maintained on a stock ration, these differences in cecal contents were not apparent. It is indicated from the results of Gall, *et al.*, that not only is there a wide variation between the nutritional requirements of various species of animals, which in certain instances can be explained on the basis of intestinal flora, but that there may also occur variations in the nutritional needs of strains of animals within one species that may be a function of intestinal biosynthesis.

It was not until the past decade that our knowledge of the various vitamins, particularly members of the water soluble B-complex, was sufficient to permit the use of highly purified, chemically defined rations, commonly referred to as "synthetic rations." According to Elvehjem (20), "Before 1940 any attempt to grow rats on a vitamin-B-complex-free ration supplemented with the crystalline vitamins then available resulted in complete failure." Within a short time it was discovered that the addition of pantothenic acid, thiamine, riboflavin, niacin, pyridoxine, and choline as the only B vitamins to purified rat rations resulted in good growth and reproduction. Elvehjem, and no doubt many other nutritionists, was not convinced that these six members of the vitamin B-complex were the only ones required by the rat, but rather were the B vitamins synthesized to the least degree by intestinal microörganisms—a view well substantiated by Mitchell and Isbell (65). Other vitamins apparently were needed by the rat but intestinal synthesis of them was so great that their presence in the ration was not a prerequisite.

Such a theory was difficult to substantiate until Marshall, *et al.* (56) reported the bacteriostatic effect of the poorly absorbed sulfonamide, sulfaguanidine, on intestinal bacteria (especially coliforms). Therefore, an approach was suggested whereby it might be determined whether or not intestinal synthesis of other factors was occurring in the rat as a result of subduing the activities of some intestinal microörganisms. The response to this suggestion was immediate, particularly by workers at the University of Wisconsin, the National Institute of Health, and the Johns Hopkins University. Within a few years' time it was discovered that incorporation of poorly absorbed sulfonamides in the ration resulted in deficiencies corrected by para-aminobenzoic acid, biotin, folic acid and vitamin K. Almost without exception, a considerable reduction in the numbers of coliform organisms accompanied the presence of certain sulfonamides in the diet. The report of Gant, *et al.*, (32) indicated that the coliform flora may gradually re-establish itself in the presence of sulfaguanidine and sulfathiazole, but Miller (62) found that neither a "sulfa-resistant" nor a "sulfa-sensitive" strain of *E. coli* was able to synthesize as much folic acid in the presence of sulfathiazole as in its absence. Evenson, *et al.* (25) found not only the coliform flora to be affected by use of sulfathiazole in rat rations, but that lactobacilli also were reduced in numbers by the presence of the drug.

For a complete review of work reported on the use of sulfonamide drugs in nutritional and bacterial flora studies it is suggested that the reader consult Evenson (24) and the excellent, comprehensive review by Daft and Sebrell (15). Undoubtedly the use of bacteriostatic agents which are not harmful to the host is becoming a useful tool in determining the extent of vitamin synthesis by intestinal microörganisms, and the exact nutritional requirements of various animals, including humans.[3] This type of study is analogous to attempts to raise animals free from intestinal organisms, although the latter would appear to be the more ideal approach to the problem of dependence of animals on synthetic products elaborated in their intestinal tracts by microörganisms. However, progress in raising sterile animals has been slow, probably because there is synthesis of as yet unidentified growth factors which must be present in rations of bacteria-free animals for them to survive and grow.

In this section, an attempt has been made to cover the more important reports leading up to the undeniable significance of intestinal microörganisms in the nutrition of the host animal. However, the literature is extremely voluminous, and a complete coverage would be unwieldy and perhaps confusing. Therefore, it is advised that the reader supplement this summary by consulting several more extensive reviews (15, 20, 21, 22, 45, 46).

THE EFFECT OF VARIOUS CARBOHYDRATES ON THE INTESTINAL FLORA AND UPON NUTRITION

As a result of the important discovery of refection (discussed in the previous section), attention was directed to the rôle of the carbohydrate component of the ration in intestinal synthesis. The effect of lactose and lactose-containing foods upon the intestinal flora of experimental animals was known for a considerable time (81) before the observation of refection. It was known that a low carbohydrate, high protein diet resulted in a flora deficient in lactobacilli and containing large numbers of coliforms and putrefying types of bacteria. By incorporation of more carbohydrate, especially lactose, and reducing the protein in the diet, it was shown that the numbers of lactobacilli would increase and putrefying microörganisms would be considerably reduced in numbers. The necessity of starch for production of refection offered a clue as to the rôle of carbohydrates in nutrition.

Mitchell (63, 64) reported that dextrin and starch supplemented with the usual components supported the best growth of rats, while maltose and sucrose were fair, and lactose poor in this respect. She found that by using mixtures of corn starch and lactose (60 per cent total carbohydrate), a ration containing these carbohydrates in a ratio of 1:1 resulted in growth as satisfactory as when all the carbohydrate in the diet was starch. Any ratio in favor of lactose resulted in

[3] It must be realized, however, that bacteriostatic agents may do more than just inhibit growth of certain vitamin synthesizing organisms in the intestines.

reduced growth responses. She also noted that as the per cent starch was increased in the ration, the per cent total carbohydrate remaining in fecal droppings decreased. Apparently lactose was less readily assimilated in the rats she used than was starch. Whether this preferential action on starch was a reflection of microbial activity, or of an insufficiency of normal alimentary lactase, or, more probably, a delicate combination of both of these factors, cannot be determined from Mitchell's work. Such information would be highly useful.

Guerrant, *et al.* (35, 36, 37, 38) made a series of studies which elaborated on Mitchell's results. They studied rats on rations deficient in vitamins "B" and "G" and observed an increased demand for these deficient factors when dextrin was replaced by sucrose, lactose, glucose, or commercial corn starch. Thus, dextrin encouraged a vitamin-synthesizing intestinal flora. These workers concluded that the difference between the carbohydrates was due to the fact that mono- and disaccharides and starch were more quickly assimilated than was dextrin; thus, with dextrin, a residual amount of carbohydrate was available for synthesizing bacteria in the lower regions of the tract (they found the greatest synthesis to occur in the cecum). To test their hypothesis, they determined the reducing intensity, expressed as milli-equivalents (ME) of glucose, of cecal contents of rats on the deficient ration with various carbohydrates. Reducing values of from 43 to 80 ME were obtained from cecal contents of rats on starch, lactose, glucose and sucrose. However, values obtained from rats on dextrinized corn starch ranged from 292 to 327 ME. These results, unfortunately, have not been further investigated, and it would be interesting to determine the necessity of utilizable carbohydrate for the elaboration of members of the vitamin B-complex by bacteria of the intestinal tract.

Morgan, *et al.* (67) studied the relationship between the type of dietary carbohydrate and deficiencies of riboflavin, vitamin B_6 (pyridoxine) and the filtrate factor (pantothenic acid) in rats. They reported that lactose favored synthesis of riboflavin and pyridoxine, corn starch favored synthesis of pantothenic acid, and sucrose favored synthesis of none of these three members of the B-complex. Rats on the deficient diet containing starch grew best although sometimes they developed a dermatitis. In a similar study (94, 95), it was found that sucrose was very effective in producing severe riboflavin and B_6 deficiencies in rats, manifested by the appearance of cataracts and a dermatitis, respectively, whereas corn starch was beneficial, although the animals grew poorly. It was further noted that rats on B-deficient rations fed feces from lactose- and dextrin-fed rats grew normally, but that feces from rats consuming any other carbohydrate were ineffective in alleviating the deficiency syndromes. The physical appearance of fecal droppings from rats fed lactose or dextrin was normal, while those from rats on sucrose, glucose or starch diets were hard, poorly formed, and small. The ceca of autopsied rats fed lactose or dextrin rations were distended and well filled, while those from animals on one of the other carbohydrate diets were contracted and rather empty. Riboflavin was isolated only from feces of rats fed lactose- or dextrin-containing rations, and it was believed that these two carbohydrates favored the development of a synthesizing flora.

Despite the universal belief that carbohydrates are essential in the ration (22), Follis and Straight (26) published a brief report upon the occurrence of adequate growth of rats on a purified ration devoid of carbohydrate. This report seems not to have been substantiated, and until it is, any further comment is reserved. Unfortunately, they reported no bacteriological studies on these animals.

Erschoff and Deuel (23) found rations containing lactose or beta-lactose to produce alopecia and diarrhea in rats on presumably "complete" diets. Corn starch, sucrose and galactose in the same rations supported good growth with no evidence of vitamin deficiencies. Their results do not entirely agree with those of other investigators already mentioned. Mannering, *et al.* (55) found diets deficient only in riboflavin to be in every way adequate for rats if the carbohydrate component were either dextrin or starch. Isocaloric replacement of the carbohydrate with lard resulted in riboflavin deficiencies as did the use of cellulose, sucrose or lactose. Their results led them to conclude, as did Guerrant, *et al.*, that incomplete digestion of dextrin or starch allows some of the carbohydrate to reach the cecum where it can be utilized by vitamin-synthesizing bacteria. On the other hand, sucrose probably is entirely assimilated before peristalsis brings the ingesta down to the large intestine and cecum, where the greater share of synthesis presumably occurs. Results indicating beneficial effects of lactose have been announced by Schweigert, *et al.* (88) who noticed that lactose served as a better stimulant for the synthesis of thiamine and riboflavin than dextrin, but that the latter was more stimulatory than sucrose.

Bartree, *et al.* (3) obtained evidence indicating the stimulatory effect of available sugar upon the intestinal flora. They found that when three pounds of glucose were fed to a cow in addition to the customary feeding of hay, the direct bacterial counts in the rumen soon increased 100 per cent over what they had been. The desirability of a simultaneous study on vitamin synthesis is indicated by these results and might provide some explanation for the differential effect of various carbohydrates upon intestinal synthesis. There is good reason to believe that the "synthesizing areas" of the intestinal tract—the cecum and the large intestine—behave similarly to such a stimulus.

Many reports in the literature indicate the superiority of dextrin over other carbohydrates in promotion of intestinal synthesis. Luckey, *et al.* (54) found that low levels of dietary folic acid were in no way impairing to chicks, provided corn meal or dextrin was the source of carbohydrate. Sarma, *et al.* (86) found dextrin to encourage pyridoxine synthesis in rats on a B_6-deficient ration; glucose and sucrose were not stimulatory. An extensive study by Skeggs and Wright (90) showed that lactose failed to promote growth or survival of rats on a complete synthetic diet. They found dextrin-fed rats to excrete appreciably more of all the B vitamins, except riboflavin, than did rats on sucrose, cerelose, lactose or corn starch. Such rats also stored more pantothenic acid than did rats on any of the other carbohydrates. Their study also embraced work on the effect of succinylsulfathiazole on rat nutrition. They found that rats developed a combined folic acid and biotin deficiency when fed succinylsulfathiazole in 2 per cent amounts no matter which carbohydrate was employed in the ration. It was shown that the drug produced a marked decrease in the numbers of *E. coli* but that neither succinylsulfathiazole nor the type of carbohydrate appeared demonstrably to alter the kinds or numbers of other bacteria in the intestinal tract. A recent study by Teply, *et al.* (97) further strengthened the already strong evidence concerning the stimulatory effect of dietary dextrin on intestinal synthesis. When fed a ration deficient in niacin and folic acid, rats ingesting dextrin as their sole carbohydrate synthesized a considerable amount of these two vitamins, especially in the cecum, but rats fed the same ration with lactose instead of dextrin developed homologous deficiency syndromes. They also found that addition of excess niacin increased the amount of folic acid recovered in the cecum; conversely, addition of excess dietary folic acid increased the amount of niacin recovered in the cecum.

Nath, *et al.* (69) have reported that the cecal contents of rats on a lactose-containing diet had greater total numbers of bacteria, more coliforms, and more lactics than cecal contents of rats on sucrose or dextrin-containing diets. Dextrin in the diet was found to favor higher numbers of coliforms in the cecal contents than sucrose. The aerobic and anaerobic plate counts, as well as the numbers of coliforms, were found to be decreased in the ceca of rats fed sucrose diets containing a high level of corn oil (28 per cent). However, supplementation of the high fat diet with reticulogen was found to counteract the inhibitory action of corn oil upon the growth of cecal microörganisms.

In an unpublished study by Krehl and Carvalho (Elvehjem and Krehl, 22), the use of dextrin in pantothenic acid-deficient diets greatly improved rat growth over that obtained with sucrose; this result could be correlated with higher levels of this vitamin in cecal contents, liver and muscle tissues. Krehl and Carvalho also found that biotin deficiencies were delayed in the rat when dextrin was used instead of sucrose as the only dietary carbohydrate "despite the fact that the dextrin-fed rats grew better, which would tend to increase the demand for biotin."

The latter work was well substantiated and enlarged upon by Couch, *et al.* (14) in their study on biotin deficiency in the chicken. A synthetic ration deficient in biotin and differing in its carbohydrate component was fed to laying pullets. The rations employed were as follows: (a) sucrose + basal, (b) dextrin + basal, (c) lactose + sucrose + basal, (d) sucrose + basal + biotin, (e) whey + sucrose + basal, and (f) a practical grain ration to serve as an additional control. Four hens were maintained on each of the six rations. It was found that the hens fed the dextrin diet synthesized adequate amounts of biotin in their intestinal tracts since their egg production was normal, the biotin content of the eggs produced was nearly equal to that of hens on the grain or "complete" synthetic diet (ration d), and hatchability of eggs from these hens was normal. In these respects, sucrose, whey, and lactose were poor. A concurrent study on these hens by Johansson, *et al.* (43) showed that birds on the sucrose ration had a fecal coliform flora almost too low to be detected, whereas dextrin-fed hens were found to have a very large coliform flora in their fecal droppings. Lactic acid bacteria were found to occur in the greatest numbers in the feces of hens fed lactose- and whey-containing diets. It seems probable that the favorable effect of dextrin in biotin deficient rations for chickens lies in the establishment of conditions in the intestinal tract favorable for synthesis of biotin. Actually, there might have been no important qualitative or quantitative differences in the intestinal flora of these birds on the various diets if one accepts the well-founded theory that dextrin is less rapidly assimilated than other dietary carbohydrates. The mere presence of significant amounts of fermentable carbohydrate in the lower intestine may be a prerequisite for vigorous biotin synthesis; if the amount of carbohydrate remaining after assimilation is too little, biotin synthesis might be hindered.

Actually, there have been few concerted attempts to design experiments from which data could be derived pointing to the exact nature of the influence of the type of dietary carbohydrate on the elaboration of nutrilites within the gastrointestinal tract. A wealth of indirect information has accumulated since the advent of synthetic rations and there is, no doubt, a significant relationship between the degree of intestinal biosynthesis and the accompanying carbohydrate in the diet. It appears, therefore, that the nature of dietary carbohydrate is very important when the degree of intestinal vitamin synthesis by microorganisms is considered. The greatest share of evidence points to the more complex carbohydrates as being the most stimulatory in vitamin synthesis, and the simplest carbohydrates as being the least stimulatory.

More informative studies similar to those of Mitchell and Isbell (65) and of Benesch (4), which have been reviewed in the first section of this paper, would be welcome and should contribute materially toward our understanding of the basic rôle of intestinal microörganisms. We know that there are numerous influences upon the types, numbers and activities of intestinal microörganisms, e.g., diet (which in itself is an extremely variable factor), pH, Eh, the animal species involved, the normal digestive activity, and possibly certain other factors (surface tension, mineral concentration, natural metabolic antagonists, synergisms, and age of the animal). Therefore, the ecological system occurring in the alimentary canal must be complex and delicately balanced, and lends itself poorly to accurate scientific study. Efforts to raise animals free from intestinal bacteria, though sometimes successful, do not necessarily prove that an intestinal flora is superfluous for proper nutrition. It must be realized that such animals are maintained on the most nutritionally adequate diet that it is possible to provide. In all probability, most animals, and human beings in particular, are rarely provided with a completely balanced diet, and apparently rely upon synthetic activities of their intestinal microörganisms to provide many of the deficient growth factors.

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